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Direct fluorimetric characterization of dyes in ancient purple codices

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Abstract

The identification of the purple dyes employed to color purple codices is an intriguing task, as sampling is seldom permitted on these precious manuscripts and thus scientists should rely only on *in situ* analytical techniques. The availability of a small sample from the 6th century *Codex Brixianus*, conserved at Biblioteca Civica Queriniana (Brescia, Italy), allowed us to perform an in depth fluorimetric survey of the purple parchment of this artwork. Preliminary *in situ* fluorimetric measurements widened previous analyses on the same manuscript as they revealed the presence of both folium and orchil in some pages. In addition, emission fluorescence and luminescence lifetime measurements were performed on the sample from *Codex Brixianus* and on a set of reference parchments dyed with folium, orchil or a mixture of these two dyes. These measurements confirmed and narrowed the results obtained with *in situ* fluorimetry, thus opening the discussion about the unusual manufacturing technique of this peculiar purple codex. Moreover, they are relevant to face other possible situations in which the two dyes have been used together to obtain the purple color.

Keywords: folium, orchil, fluorimetry, lifetime, ancient manuscripts, purple

1. Introduction

The investigation of works of art represents an intriguing task for scientists. As the tests may deal with precious and fragile artefacts, the analyst should get as much information as possible about the constituting materials and the state of conservation without threatening the integrity of the object. The topic pushed the research towards non-invasive portable analytical techniques, which do not require sampling and enable an *in situ* investigation of the artefact [1]. Nevertheless, non-invasive procedures show both strong and weak points. In particular, when natural dyes are considered, the obtained information may in some cases be unsatisfactory in order to achieve a thorough characterization of the dyes [2-4].

For this reason, micro-invasive techniques are still widely employed when the presence of one or more natural dye is hypothesized in an artwork, being High Performance Liquid Chromatography (HPLC) coupled with different detection methods (mainly Diode Array Detection and Mass Spectrometry) the election technique [5,6]. Moreover, Surface-Enhanced Raman Spectroscopy (SERS) has been proposed in the last ten years, in order to reduce the sample amount down to the microscale level [7-10]. Sampling restrictions become really binding when very precious and rare objects as purple codices are considered. The characterization of the dyes employed to color these peculiar manuscripts is still a challenge. Purple codices are manuscripts written in silver and golden inks on purple-colored parchments produced from the Late Antiquity to the Middle Ages to be owned by Kings or Emperors [11]. Only a modest number of these amazing works of art still survive in libraries or archives. Ancient treatises about art and painting techniques give a little information about how the parchment was colored (see [12,13] and references therein), explicitly indicating the use of orchil, whereas Tyrian purple had never been cited when dealing with

parchment dyeing. Despite the indirect evidence of the presence of Tyrian purple based on the detection of the bromine peak in XRF spectra from purple parchments [14], the use of orchil (e.g. the dye that slowly forms by fermentation upon treating different genera of lichens in a basic aqueous medium) has been hypothesized [15-17]. Moreover, it has been shown [13] that the evidence of bromine is not anymore a confirmation that Tyrian purple is present in an artwork, being this element also contained in some lichen species. Recent analyses by means of non-invasive or micro-invasive techniques (e.g. portable fluorescence, lifetime measurements, fibre optics reflectance spectroscopy, subtracted-shifted Raman spectroscopy, LC-qTOF-MS) would also support the use of orchil [13,18-22]. In addition, the use of folium (obtained from the plant *Chrozophora tinctoria* (L.) A. Juss.) was hypothesized in an early work by Thomas and Flieder [23] and later by Roger [24,25] and Aceto [13,22]. Recently, the possible use of orchil or folium has been suggested in one manuscript considered for the scientific investigation with non-invasive spectroscopic procedures [12].

Orchil has been characterized in the 1950s-1960s, in the extensive work by Musso and co-workers [26,27 and references therein] who elucidated the formation, the structure and the properties of its numerous chemical components. In particular, the lichens employed to obtain orchil contain orsellinic acid depsides, which after hydrolysis are decarboxylated to the colorless orcinol. Orcinol is, in turn, oxidised to orcein with the introduction of ammonia [26]. These authors identified fourteen nitrogen-containing dyes plus two yellow-brown nitrogen-free compounds. Moreover, a study of orcein and its metal complexes in aqueous solution underlined that the absorption band in the visible region of orcein is due to the intramolecular charge transfer from the orcein moiety and the imine nitrogen to the quinone nucleus of the molecule [28]. Despite the work that has been done on the characterization of folium [29], at the best of the authors' knowledge the chemical structure of its purple components has not been published yet and the research is in progress on this topic.

Besides the already cited works on purple codices, other authors have achieved the detection of orchil in various samples from the cultural heritage domain. In particular, in its pioneering work, Wallert [30] suggested the possibility of identifying different species of orchil lichens by means of fluorimetry on samples dissolved in an aqueous solution acidified with sulfuric acid. Furthermore, *in situ* fluorimetry has been proposed to investigate orcein in different solvents and substrates of interest for the cultural heritage (e.g. silk, wool) [31] and allowed the identification of orchil on tapestries [32]. This technique proved to increase its diagnostic ability when coupled to luminescence lifetime measurements, as other dyes - such as cochineal - may exhibit similar fluorescence spectra but very complex and notably different fluorescence lifetimes [33]. The diagnostic information for the detection of orchil and folium has been recently increased thanks to the investigation of raw materials and of painted and dyed parchments by means of a number of non-invasive and micro-invasive techniques (e.g. FT-IR, FT-Raman, fibre optic reflectance spectrophotometry, portable spectrofluorimetry, X-ray fluorescence spectrometry) [13]. Besides this recent work, only two congress papers deal with the characterization of folium [34,35]. In these cases, the use of 3D fluorescence for the identification of a number of dyes, including folium and orchil, has been discussed.

In the present work, fluorimetric techniques have been employed for the characterization of the purple parchment. As a whole, the techniques based on molecular luminescence proved to be valuable for the identification of dyes and inorganic pigments in ancient materials [3,4,36]. Here, their suitability for discriminating between orchil and folium - and for the detection of these two dyes when they are employed together in a dyed or painted parchment - is tested. The investigation was set up starting from the work by Aceto *et al.* [12] on the purple manuscript known as the *Codex Brixianus*, a precious 6th century codex conserved at the Biblioteca Civica Queriniana in Brescia (Italy). According to *in situ* measurements

performed by means of UV-visible Fiber Optics diffuse Reflectance Spectroscopy (FORS), it was suggested that orchil or folium were most probably present in the parchment. As the investigation was not conclusive in discriminating between the two dyes, the topic is further considered here. Firstly *in situ* measurements by means of portable fluorimetry were set in order to obtain preliminary information about the dyes that can be found in the different pages of this precious manuscript. Secondly small fragments of reference parchments dyed with orchil, folium or a mixture of them were investigated by emission fluorescence and luminescence lifetime measurements. Then a small sample from the *Codex Brixianus* was also considered in order to deepen the results previously achieved [12] and to check whether the comparison with the data obtained on the reference parchments dyed in the laboratory would deepen the knowledge on the coloring materials and procedures.

2. Material and methods

Orchil was obtained following the procedure indicated by Kok [37], i.e. upon fermentation in 30% v/v ammonia of the scraps of the lichen *Roccella tinctoria* DC. The lichen was provided by Isabella Whitworth (Dorset, UK), who gathered it in the Canary Islands. Folium was obtained by suspending in water for three hours at room temperature the pericarps of the fruits of *Chrozophora tinctoria* (L.) A. Juss. from Umbria (Italy) provided by L. Menghini (Università "G. d'Annunzio" di Chieti-Pescara). The extract was then filtered and allowed to dry. Parchment was handmade from goat skin by an artisan in Piemonte (La Pergamena di Bonino Ivano, Ciriè, Italy) following medieval procedures.

2.1 Preparation of reference parchments

Model samples of parchment dyed with folium, orchil or both folium and orchil were prepared as follows. Firstly the parchment was gently scraped with a fine polishing paper in order to make it more suitable to soak in the dyeing bath. Then, a solution of the powdered dye and alum was prepared at room temperature. The addition of alum was necessary to permanently fix the color on the parchment and it was also needed for orchil, although it is known as a direct dye on textiles. The weight of alum was 30% with respect to the dry weight of the parchment, as well as that of orchil powder, while, due to its weaker dyeing yield, the weight of folium powder was 100% with respect to the parchment. The parchment was left in the solution for 24 hours and then allowed to dry in the dark. In order to obtain samples dyed with both dyes, some parchments were subsequently put into the two baths, or in a bath obtained by mixing the two dyes. Five samples of reference parchment were obtained: one dyed only with folium (henceforth PF), one dyed only with orchil (henceforth PO), one dyed with both orchil and folium in the same dyeing bath (henceforth PM), one dyed first with folium and then with orchil (henceforth PFO) and a last one dyed first with orchil and then with folium (henceforth POF). In all instances an intense color saturation was obtained. This intense color may represent a limit for the fluorimetric investigation, nevertheless it mimics the real situations that can be encountered when investigating purple codices.

2.2 Codex Brixianus

A sample (2x2 mm) was obtained from *Codex Brixianus*, already investigated by means of non-invasive analytical techniques [12]. This sample exhibited a darker and a lighter side (Figure 1 a-b); both of them were considered in the fluorimetric characterization performed in the present work.

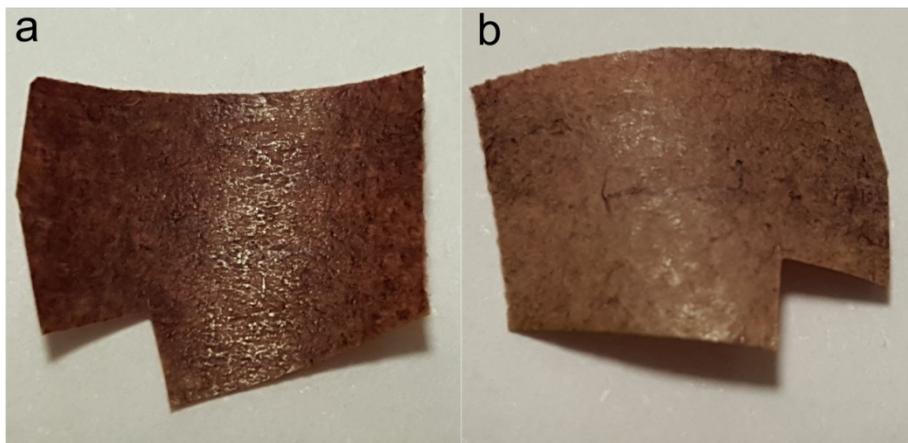


Figure 1: the sample taken from *Codex Brixianus*: darker side (a) and lighter side (b); the longer side of the sample measures about 2 mm.

2.3 Instrumentation

2.3.1 Portable fluorimetry

An Ocean Optics (Dunedin, Florida, USA) Jaz model spectrophotometer was employed to perform *in situ* measurements of the purple pages of *Codex Brixianus*. The instrument is equipped with a 365 nm Jaz-LED internal light source; a QF600-8-VIS/NIR fiber fluorescence probe is used to drive excitation light on the sample and to recover emitted light. The spectrophotometer is working in the range 191–886 nm; according to the features of monochromator (200 μm slit width) and detector (2048 elements), the spectral resolution available is 7.6 nm calculated as FWHM. The investigated area on the sample is 1 mm diameter. In all measurements the sample-to-probe distance was kept constant to 12 mm (corresponding to focal length); the probe was designed so as to exclude contributions from external light. To visualize the sample, the probe was equipped with a USB endoscope. The instrumental parameters were as follows: 4 s integration time, 3 scans, for a total acquisition time of 12 s for every spectrum. The system is managed with the SpectraSuite software under Windows 7.

2.3.2 Bench-top fluorimetry

Steady state emission and excitation spectra were recorded by a Horiba Scientific Fluorolog spectrofluorimeter equipped with a 450 W Xenon lamp, double-grating excitation and emission monochromators (2.1 nm mm^{-1} dispersion; 1200 grooves per mm) and a Hamamatsu R928 photomultiplier tube. Emission and excitation spectra were corrected for source intensity (lamp and grating) and emission spectral response (detector and grating) by standard correction curves.

Time-resolved measurements were performed using the time-correlated single-photon counting (TCSPC) technique, with a pulsed excitation source, NanoLed, at 562 nm (Horiba). The excitation source was mounted directly on the sample chamber at 90° to a double-grating emission monochromator (2.1 nm mm^{-1} dispersion; 1200 grooves per mm) and signals were collected using an IBH Data Station Hub photon counting module. DAS6 decay analysis software was used for raw data fitting and lifetime calculation.

3. Results and discussion

3.1 *In situ* fluorimetry

In a previous paper on *Codex Brixianus* [12] the obtained FORS spectra allowed us to identify the presence of orchil or folium, but were not conclusive in discriminating between them. A further larger analytical campaign focused on this purple parchment was therefore performed by *in situ* fluorimetry. The spectra

obtained in most of the different pages of the codex showed features attributable mostly to orchil, according to an emission peak at ca. 624 nm. Nevertheless, some pages (namely ff. 63v and 65v) presented two different hues: more reddish in the side part, more bluish in the central part, the one under the text. Indeed, the emission spectra of these pages presented the features of orchil in the reddish part, but the features of both dyes were detectable in the central part (Figure 2), thus suggesting a possible combination of orchil and folium to obtain the final color.

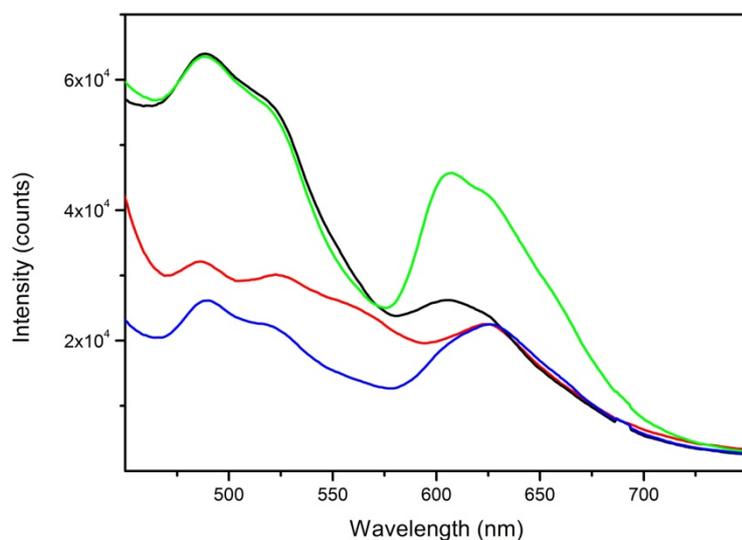


Figure 2: Fluorimetry spectra obtained with portable instrumentation on a reference parchment dyed with folium (black line), a reference parchment dyed with orchil (red line), page 55 of *Codex Brixianus* (blue line) and page 88 of *Codex Brixianus* (green line).

3.2 Fluorimetry

Steady state emission spectra of orchil and folium were measured on aqueous solutions having absorbance ca.0.1, in order to avoid artefacts due to inner filter effects or quenching phenomena. Emission spectra are reported in Figure 3 and the wavelength values of emission maxima are reported in Table I for the sake of easy comparison. The emission spectrum of orchil is characterised by a broad band dominated by a maximum at 592 nm, as previously reported for natural orcein by Clementi *et al.* [31]. Beside a similar maximum wavelength (595 nm), the broad emission band of folium also displays a bathochromic shoulder at ca. 640 nm. As expected, the emission spectra of a solution containing both orchil and folium are characterized by a spectral profile which is somewhat the combination of those of the two dyes: the broad band is dominated by a maximum at 594 nm, with a weak shoulder at ca. 635 nm. Due to the close similarity of the emission spectra of the two components, it appears difficult to identify the contribution of each dye. Furthermore, as it is known that the interaction with a solid matrix can induce modifications in the spectroscopic features and that the different molecules present in folium and orchil extracts can be adsorbed with different efficiency on a parchment, the five model samples were considered before approaching the characterization of the *Codex Brixianus*.

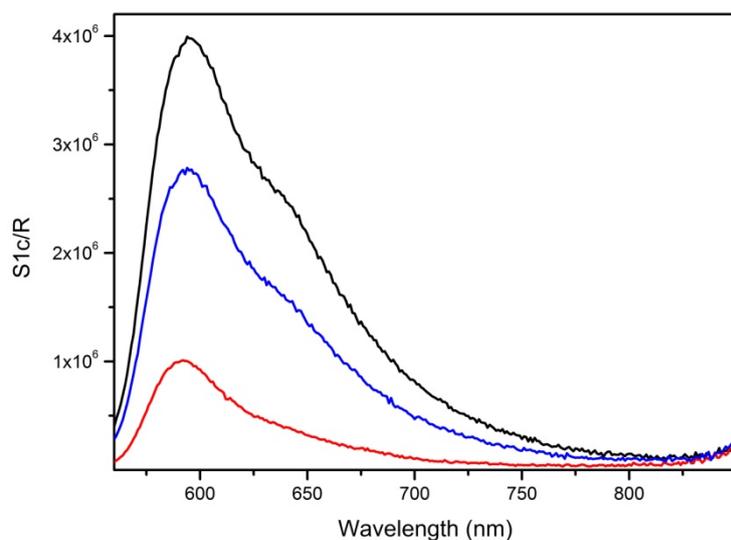


Figure 3: Steady state emission spectra of aqueous solutions of folium (black line), orchil (red line) and folium:orchil 1:1 (v/v, blue line).

The emission spectra measured on model parchments are reported in Figure 4; the wavelength values of emission maxima are reported in Table I.

Table I - Emission maxima of folium and orchil detected in solution and on dyed parchments. PO was dyed only with orchil, PF was dyed only with folium, PM was dyed with orchil and folium in the same dyeing bath, POF and PFO were dyed first with orchil and then with folium and first with folium and then with orchil, respectively.

| | | EMISSION WAVELENGTH | | |
|-----------------------------|--------|-------------------------|-------------------------|-------------------------|
| | | Max 1 (λ , nm) | Max 2 (λ , nm) | Max 3 (λ , nm) |
| AQUEOUS SOLUTIONS | Orchil | 592 | - | - |
| | Folium | 595 | 640 (sh) | - |
| | Mix | 594 | 635 (sh) | - |
| REFERENCE PARCHMENTS | PO | 625 | 645 (sh) | 730 (vw-sh) |
| | PF | 608 | 640 (sh) | 725 (sh) |
| | PM | 616 | 640 | 730 (sh) |
| | POF | 615 (sh) | 645 | 730 (sh) |
| | PFO | 630 (br) | - | 726 (sh) |

sh, shoulder; vw-sh, very weak shoulder; br, broad

The direct comparison of the spectral profiles of the dyes in solution and on the parchment highlights that the dyeing process severely affects the shape and the position of the emission peaks. In the case of PO, the emission maximum shows a red-shift of about 30 nm with respect to the spectrum of the orchil solution, and two poorly resolved shoulders appear at 645 nm and at ca. 730 nm (very weak). The shift of the emission spectrum and the overall broadening due to the change in the relative intensities of the various components can be interpreted as the result of the interplay of different factors.

Firstly, the observed shift may be the consequence of the intense absorption, which is due to high color saturation. This hypothesis is confirmed by the comparison of the emission spectra measured on various areas of the parchment characterised by different color intensities (Figure S1 in the Supplementary Material file). By considering the first two components, at 625 nm and ca. 645 nm, we can observe that the areas with lighter color are characterized by a predominance of the shorter wavelength component, whilst in the spectra of darker areas the two components are hardly identified, due to the partial reabsorption of the shorter wavelength component.

Furthermore, changes in the relative intensities of the emission spectrum could be also interpreted as a direct consequence of the interaction with the parchment surface, which can cause a modification of the probability of transitions from the ground vibro-electronic state to different vibrational levels of the excited electronic states, as well as provide different solvation to the dye molecules with consequent shift and modification of the spectral features.

Finally, being orchil a complex mixture of phenoxazones (such as hydroxyl-orceins, amino-orceins and amino-orceinimines [26]) which can exhibit different affinity for the parchment, the different spectral features of the PO with respect to the orchil solution could arise from a selective enrichment of the parchment in some of the components among those of the raw material dissolved in the dyeing bath.

The presence of the maximum at about 625 nm has been already observed in previous works both in the case of orchil on parchments and on textiles [12,13,22,33], although in some cases a feature at 640-650 nm [31] is reported. The variability of the maximum wavelength detected for orchil on different substrates could be related to different starting materials and preparation procedures, which led to samples with different color saturation and, at the same time, could be interpreted as a confirmation of the various interactions that take place between the several components of orchil and substrates of different nature such as parchment, silk or wool.

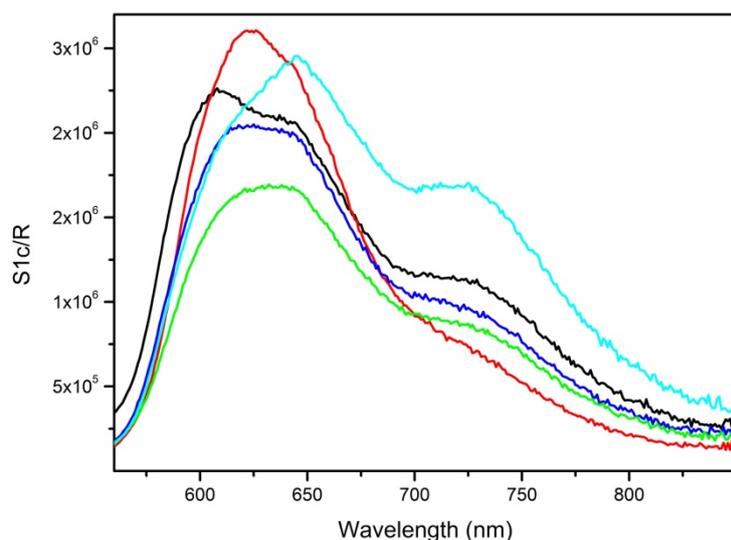


Figure 4: Steady state emission spectra of parchments dyed with folium (PF - black line), orchil (PO - red line), a mix of folium and orchil (PM - dark blue line), folium then orchil (PFO - green line), and orchil then folium (POF - light blue line).

Concerning PF, a red shift of about 15 nm of the emission spectra with respect to the spectra of folium in solution was observed, whilst the component at ca. 640 nm remained almost unchanged; an additional shoulder at ca. 725 nm appeared. The more limited red shift of the main emission wavelength observed in this case with respect to orchil suggests that the folium dyed parchment is less affected by color saturation and re-absorption effects, as confirmed by the lower variability observed when comparing spectra acquired on parchment areas characterized by different color intensities (Figure S2 in the Supplementary material file). Previous works by Aceto *et al.* [12,13,22] on folium-dyed parchments reported emission wavelengths of 595 nm (main peak) and of 625 nm (shoulder); as previously stated, changes in preparation procedures, as well as different detection set-up, can result in the variability observed in the literature. In particular, if no spectral correction is adopted for the photomultiplier's spectral sensitivity, an underestimation of the red-wavelength components occurs. This fact could possibly explain why the shoulder at 725 nm has not been detected before.

The parchments dyed with both folium and orchil exhibit a broad emission band composed by a first region (500-700 nm) where the overlapping of the orchil and folium emissions (as well as the presence of color saturation effect) give rise to a double peaked maximum, and a second region at longer wavelengths (> 700 nm), where the contribution of folium is clearly distinguishable. Beside some small variations in the relative intensities, the parchment PM and the parchment PFO appeared to be quite similar. On the contrary, in the parchment POF, the higher wavelength component ascribable to folium is more accentuated. However, this cannot be taken as a quantitative indication of a higher folium content, as the apparent higher intensity can be induced by a more severe color saturation/re-absorption effect, present with similar extent on both lighter and darker areas of the parchment (Figure S3 in the Supplementary material file). Furthermore, the overall higher emission intensity of POF with respect of PFO suggests that the order of dyes applications has some influence on the total loading of the parchment.

In summary, the different position of the first emission component and the presence of an intense shoulder at ca. 730 nm in the case of folium, permit to easily distinguish folium and orchil on parchment when they are employed alone. The situation is much more complex when both dyes are present on the same parchment, but, as discussed above, some features of folium and orchil are anyway distinguishable.

As it can be seen in Figure 1, the two sides of the sample from *Codex Brixianus* (hereafter CB) were not identical. Both sides were considered when measuring emission spectra and they were referred to as "darker side" (CB-DS) and "lighter side" (CB-LS); the collected spectra are reported in Figure 5 and the wavelength values of emission maxima are summarised in Table II.

Table II - Emission maxima of the sample from *Codex Brixianus*.

| | | EMISSION WAVELENGTH | | |
|------------------------|--------------|---------------------|---------------|---------------|
| | | Max 1 (λ, nm) | Max 2 (λ, nm) | Max 3 (λ, nm) |
| CODEX BRIXIANUS | Darker side | 630 (sh) | 645 | 730 |
| | Lighter side | 630 | 645 (sh) | 735 (vw-sh) |

sh, shoulder; vw, very weak

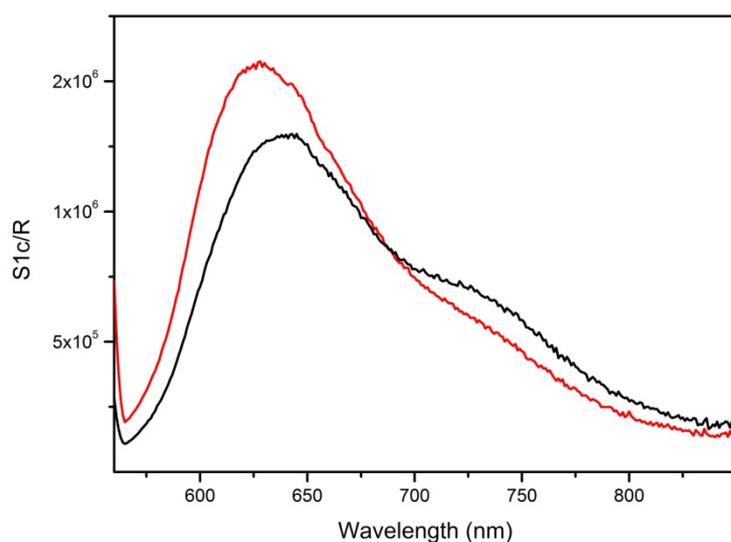


Figure 5: Steady state emission spectra of the sample from *Codex Brixianus*: darker side (CB-DS - black line) and lighter side (CB-LS - red line).

Fluorescence spectra measured on the CB sample presented three components at 630 nm, at about 645 nm and at 730-735 nm (Table II). Their relative intensities vary according to the color saturation (Figure 5). In particular, in the darker side of the parchment (Figure 5, black spectrum) the component at about 645 nm appears as an intense maximum, while at 630 nm a weak shoulder is visible. On the contrary, in the lighter side (Figure 5, red spectrum) the maximum at 630 nm is predominant, the component at about 645 nm is a shoulder and the intensity of the third maximum at about 730 nm is weaker.

Based on what previously reported by Aceto *et al.* [12,13] and by comparing the spectral profile of CB and of the reference parchments, we can hypothesize that both orchil and folium were used for dyeing the CB, even though we cannot say which was the specific production technique, that is whether the dyes were mixed or superimposed.

Due to the high complexity of the system, in order to try to solve the contribution coming from the two dyes in the fluorescence spectra of each parchment with a deconvolution approach, we calculated the derivative of each spectrum in order to isolate the different contributions. Spectra derivatization is a well-known method of separation of useful signals from noised data [38] and it ideally allows identifying a narrow band which is overlapped or completely hooded by a broad one. As we know that the spectra of the parchment are a sum of wide bands, we used second derivative; generation of high order derivatives, in fact, would result in suppression of the intensity of wide bands and in magnification of narrow ones [38,39]. The wavelength values obtained as minima in the second derivative and corresponding to hypothetical emission maxima are reported in Table S1 in the Supplementary material file, and they were used as input for the deconvolution of the spectra. The results of the deconvolution are reported in Figure S4-S10 in the Supplementary material file. Interestingly, emission spectra of CB resulted to be deconvoluted in a pattern of curves that is very close to the pattern fitting the parchments dyed first with folium and subsequently with orchil, whilst the pattern of curves obtained for the parchment PM does not resemble the CB. This finding supports our hypothesis that both orchil and folium were used for the dyeing of CB, and gives indication that that they were possibly used in a sequence and not in a mix. Moreover, there is also a good

affinity between the deconvolution pattern of CB and PO and this is in agreement with preliminary measurements with *in situ* fluorimetry, which accounted for a predominant presence of orchil.

3.3 Lifetime measurements

The analysis of fluorescence lifetimes represents a further powerful tool for the discrimination between different components present in a mixture, especially when they are characterized by similar spectral properties, as in the case of folium and orchil. Fluorescence lifetime values measured: i) on aqueous solutions of the dyes; ii) on the model parchments; and iii) on the fragment of the *Codex Brixianus* are reported in Table III.

Although in this kind of systems lifetimes distribution is made more complicated by the complex molecular composition of the dyes extracts, it is nevertheless possible to highlight some points and draw some interesting conclusions. Both folium and orchil are characterized by fluorescence decays that are well fitted by bi-exponential functions; in both cases the population characterized by the shorter lifetime (1.36 ns in the case of folium and 1.56 ns in the case of orchil) resulted to be the more abundant. When the two dyes are mixed in solution, the resulting decay trace is fitted by a three-exponential function and a small fraction of molecules exhibiting a longer lifetime (6.49 ns) arises. The fact that in the mix solution the individual lifetime of each species is no more clearly distinguishable can be due to the mutual interactions that can take place among dyes molecules and to self-absorption phenomena.

Table III - Fluorescence lifetime values of dyes in solution, dyed parchments and *Codex Brixianus*

| | SAMPLE | τ_1 (ns) / abundance | τ_2 (ns) / abundance | τ_3 (ns) / abundance | χ^2 |
|-------------------------|-----------------|------------------------------|------------------------------|------------------------------|----------|
| AQUEOUS SOLUTIONS | Folium | 1.36±0.02 / 87% | 4.00±0.02 / 13% | | - 1.02 |
| | Orchil | 1.56±0.01 / 85% | 3.13±0.015 / 15% | | - 1.02 |
| | Folium - Orchil | 0.96±0.012 / 75% | 2.49±0.01 / 23.3% | 6.49±0.03 / 1.7% | 1.08 |
| REFERENCE PARCHMENTS | PF | 1.36±0.015 / 75% | 4.02±0.008 / 25% | | - 1.04 |
| | PO | 0.54±0.008 / 26% | 2.34±0.05 / 57% | 7.08±0.02 / 17% | 1.10 |
| | PM | 0.23±0.01 / 55% | 1.23±0.03 / 22% | 4.22±0.025 / 23% | 1.05 |
| | POF | 0.69±0.03 / 87% | 2.72±0.02 / 11.3% | 5.92±0.03 / 1.7% | 1.02 |
| | PFO | 0.71±0.02 / 86.6% | 2.65±0.04 / 11.9% | 6.37±0.05 / 1.5% | 1.05 |
| CODEX BRIXIANUS | CB-LS | 0.60±0.03 / 74% | 2.24±0.02 / 22% | 7.2± 0.06/ 4% | 1.13 |
| | CB-DS | 0.59±0.02 / 71% | 2.24±0.05 / 25% | 7.27±0.05 / 4% | 1.12 |

PF presents the same lifetime values of folium in solution, but with different relative abundances. On the contrary, the lifetime distribution of orchil resulted severely modified upon the interaction with the parchment. In fact, the decay trace of PO is well fitted by a three-exponential function and the resulting lifetimes values are different both in term of absolute values and in term of abundances with respect to the values observed in solution. In particular, we assist to a decrease in the lifetime values upon interaction with the parchment, with modification of the relative abundances (from 1.56 ns / 85% to 0.54 ns / 26% and from 3.13 ns / 15% to 2.34 ns / 57%). Furthermore, a population characterized by longer lifetime (7.08 ns) and accounting for ca. the 17% of the total arises. These results are in accordance with previously published data, as a lifetime of 2.5 ns is reported for a parchment dyed with orcein [33], while a 8th century parchment dyed with orchil accounts for a 2.4 ns lifetime [19], corresponding to the more abundant species

reported in Table III for PO. PM is characterized by three lifetimes values, all the three are shorter than the corresponding values observed in solution and characterized by different relative abundances. The significant differences observed between lifetime values (and relative abundances) measured in solution and those characteristic of the dyed parchments, rely on different parameters. First of all, as already mentioned, the complex composition of the dyes extracts plays an important role. The different components of the extract, in fact, can be selectively adsorbed on the parchment, thus resulting in the enrichment of the extract of some peculiar components that, beside a similar spectroscopic behavior in term of absorption/emission band position, can display different lifetime values. Furthermore, fluorescence lifetimes are extremely sensitive to the nature of the environment. As a consequence, the change in chemical environment due to the adsorption on the parchment, as well as aggregation phenomena that are negligible in solution but very probable in the solid state, can severely affect the lifetime distribution of each dye.

In the case of POF and PFO, whatever the dyeing order, the resulting lifetimes distribution is roughly the same. The decay traces of both POF and PFO parchments are well fitted by a three-exponential decay and the two shorter components (ca. 0.7 ns and ca. 2.7 ns) could be assigned to a combination of the contribution from both folium and orchil. The third longer value is probably mainly ascribable to orchil and the reduced relative abundance with respect to PO is due to the fact that also folium is present. As already stated, the small differences observed - when comparing the lifetimes absolute values characterizing POF and PFO parchments, as well as the differences found when comparing orchil and folium lifetimes in PO and PF or in PFO and POF - rely on the high complexity of the systems. In fact, the different dyes molecules on the parchment sense different environment when adsorbed on the raw parchment or on an already dyed parchment. This variable should be intended as a factor that further increases the complexity of the studied systems.

The CB sample was measured on both the darker and the lighter side; in both cases the decay trace is well fitted by a three exponential function and the resulting lifetime distribution is very close, both in term of absolute values and relative abundances, to the results given by the parchments PFO and POF, with the shorter lifetime being the more abundant and the longer one the less abundant. At a first sight, a close similarity could be found between CB samples and PO parchment; nevertheless, the very small abundance of the longer lifetime, as well as the differences in the relative abundances of the three lifetimes, suggest that orchil should not be the only species present on the manuscript and that folium should be adsorbed on the same parchment, thus perturbing the lifetime values relative abundances.

These results allowed us to confirm the contemporaneous presence of both folium and orchil in the medieval sample and to ascertain that the dyes were superimposed by subsequent baths and not mixed in a same dyeing bath.

4. Conclusions

The fluorimetric behavior of orchil and folium in aqueous solution and dyed on parchment has been investigated in this work. The results highlighted the relevant role of the substrate, which strongly affects the response of the target molecules. Moreover, this research has shown that also the experimental set-up may bias the spectroscopic response. As a matter of fact, data obtained with portable instrumentation proved to be fairly comparable with those acquired with a bench-top spectrofluorimeter. However, it is worth noting that laboratory measurements on a sample taken from a page of *Codex Brixianus* gave a more robust interpretation of the results obtained with portable molecular fluorescence, e.g. that orchil and

folium are both present in some pages of this precious manuscript. In more detail, the fluorimetric data obtained with the laboratory equipment lead us to hypothesize that the purple color was obtained by superimposing the two dyes, rather than mixing the two dyes before coloring the parchment. Lifetime measurements were crucial to support this suggestion. This result is an important step towards a deeper knowledge about the manufacture of purple codices, but it also opens the discussion about the peculiarity of *Codex Brixianus*, which is, at present, the sole codex whose pages are colored with two different purple dyes [15-25]. An extensive analytical campaign on purple codices is required as it would allow gaining information about the dyes employed on a wider number of manuscripts and thus would confirm or deny the uniqueness of the coloring of *Codex Brixianus* by possibly linking its peculiar composition to a specific production centre.

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Figure captions

Figure 1: the sample taken from *Codex Brixianus*: darker side (a) and lighter side (b); the longer side of the sample measures about 2 mm.

Figure 2: Fluorimetry spectra obtained with portable instrumentation on a reference parchment dyed with folium (black line), a reference parchment dyed with orchil (red line), page 55 of *Codex Brixianus* (blue line) and page 88 of *Codex Brixianus* (green line).

Figure 3: Steady state emission spectra of aqueous solutions of folium (black line), orchil (red line) and folium:orchil 1:1 (v/v, blue line).

Figure 4: Steady state emission spectra of parchments dyed with folium (PF - black line), orchil (PO - red line), a mix of folium and orchil (PM - dark blue line), folium then orchil (PFO - green line) and orchil then folium (POF - light blue line).

Figure 5: Steady state emission spectra of a sample from *Codex Brixianus*: darker side (CB-DS- black line) and lighter side (CB-LS - red line).

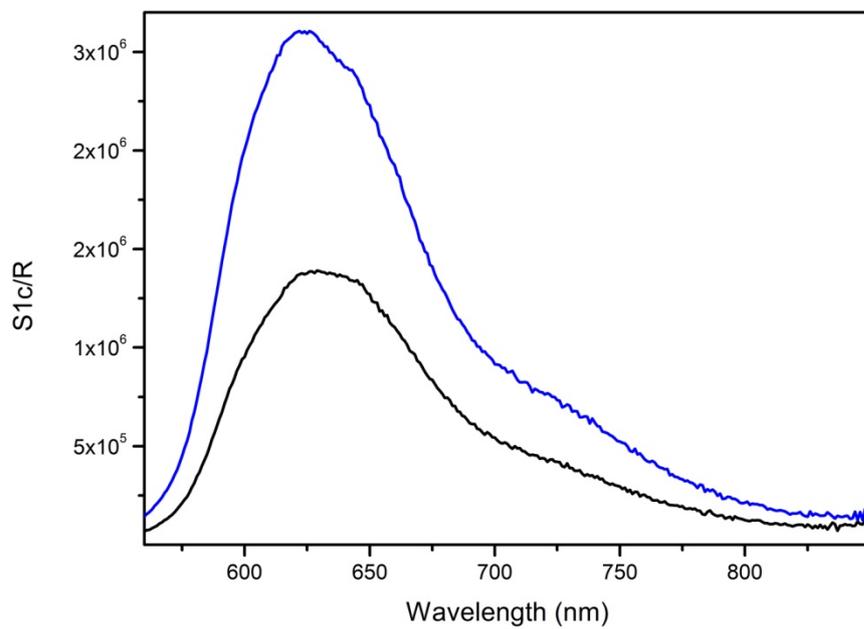


Figure S1: Steady state emission spectra of the parchment dyed with orchil (PO) obtained on a lighter (blue line) and a darker area (black line).

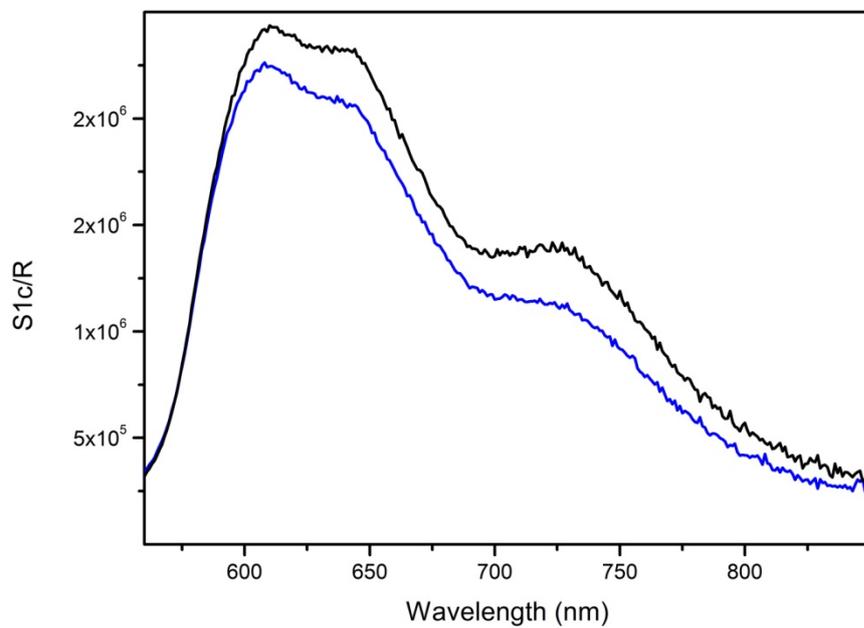


Figure S2: Steady state emission spectra of the parchment dyed with folium (PF) obtained on a lighter (blue line) and a darker area (black line).

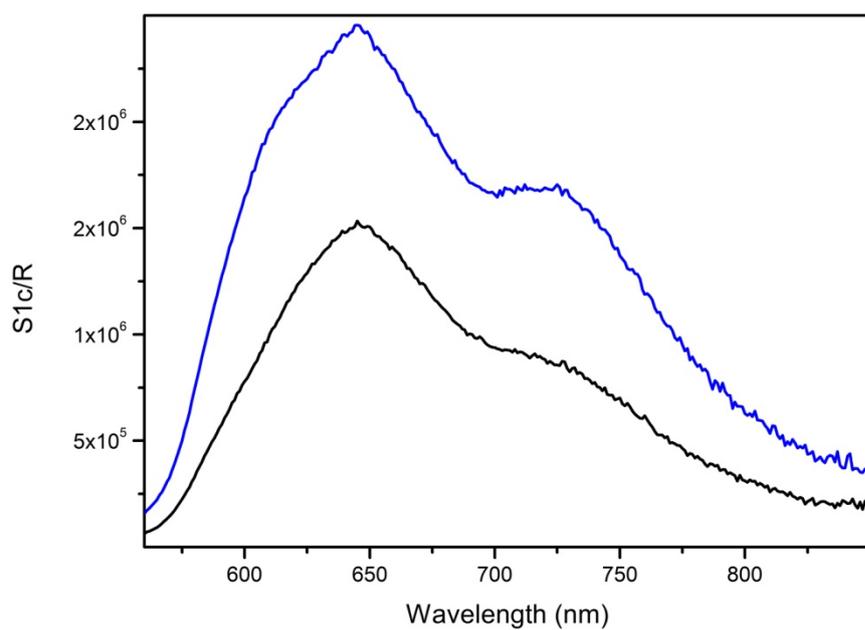


Figure S3: Steady state emission spectra of the parchment dyed with orchil then folium (POF) obtained on a lighter (blue line) and a darker area (black line).

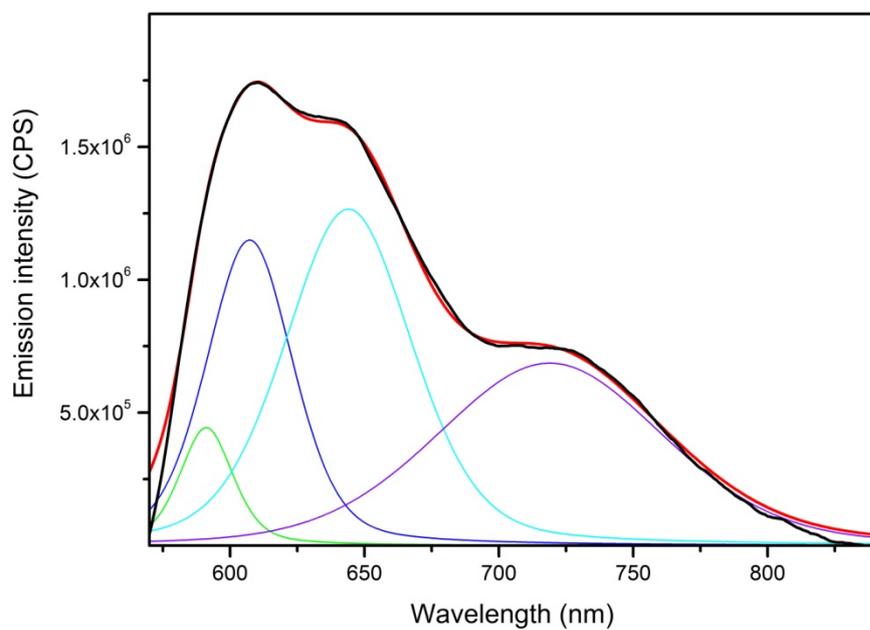


Figure S4: Deconvolution of the steady state emission spectra of the parchment dyed with folium (PF - black line).

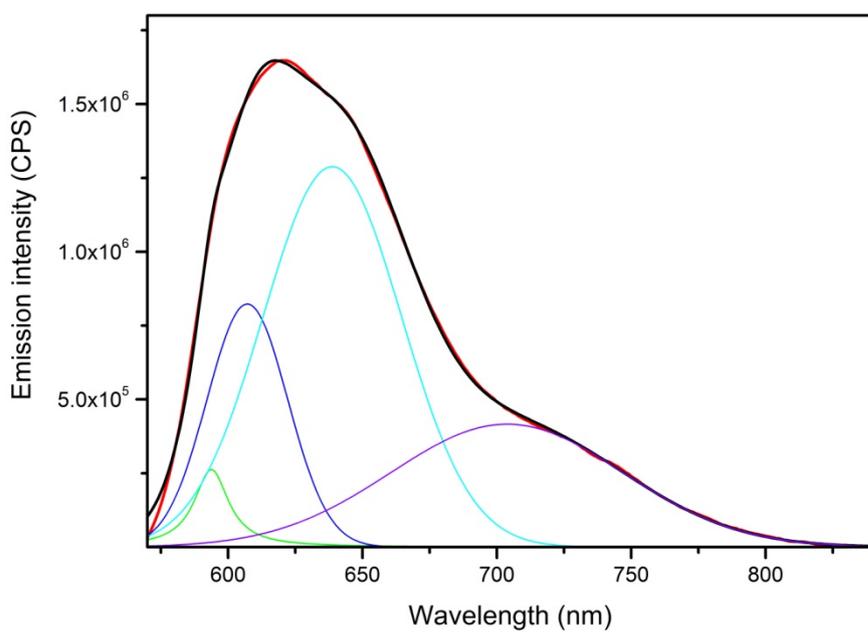


Figure S5: Deconvolution of the steady state emission spectra of the parchment dyed with orchil (PO - black line).

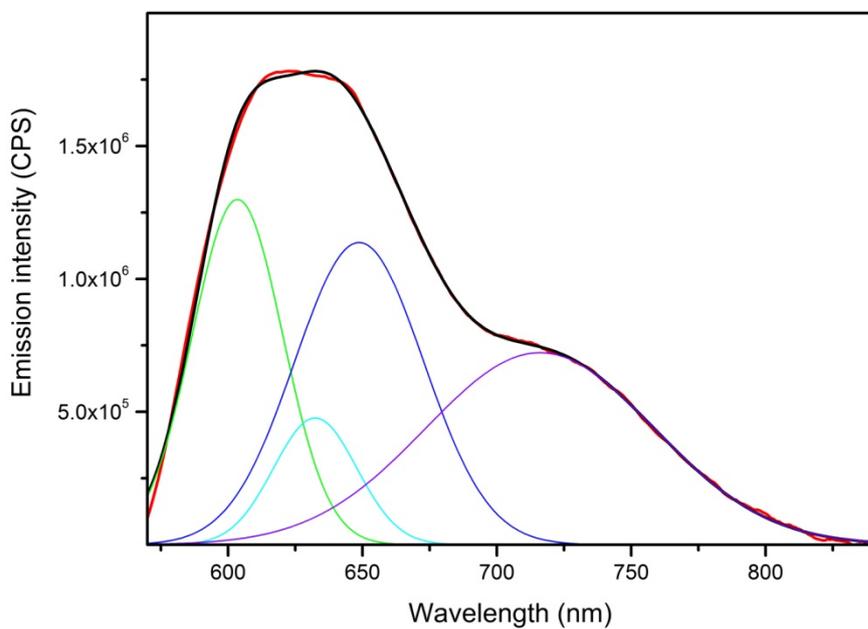


Figure S6: Deconvolution of the steady state emission spectra of the parchment dyed with a mix of folium and orchil (PM - black line).

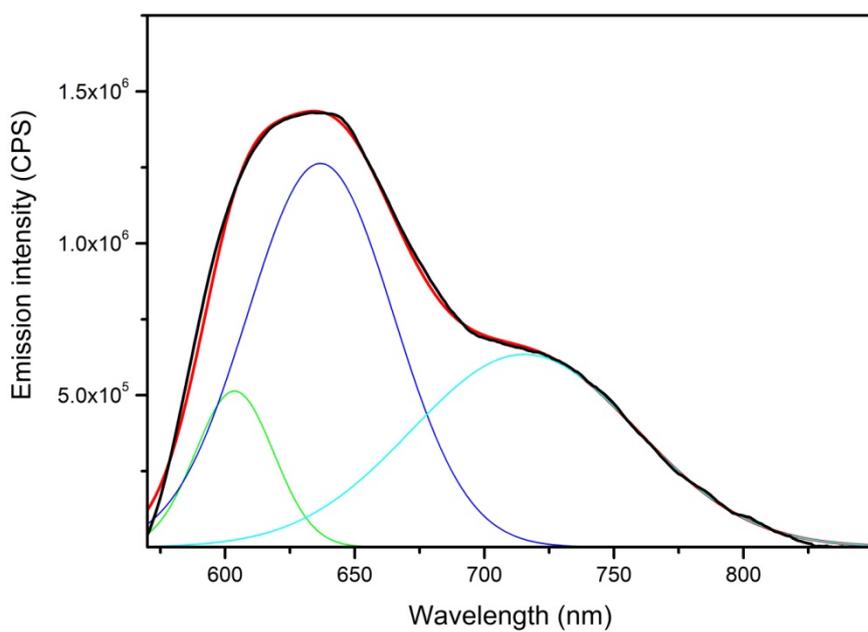


Figure S7: Deconvolution of the steady state emission spectra of the parchment dyed with folium then orchil (PFO - black line).

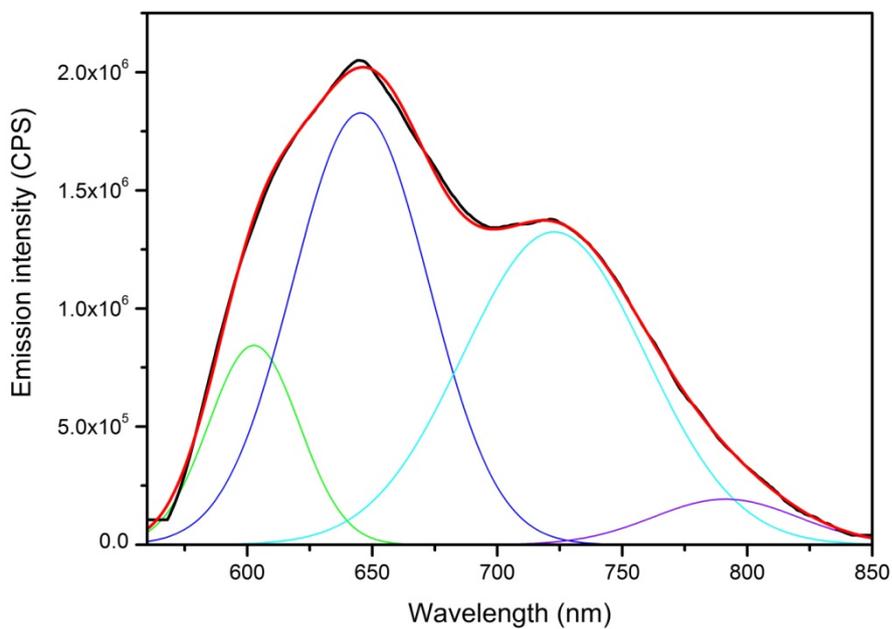


Figure S8: Deconvolution of the steady state emission spectra of the parchment dyed with orchil then folium (POF - black line).

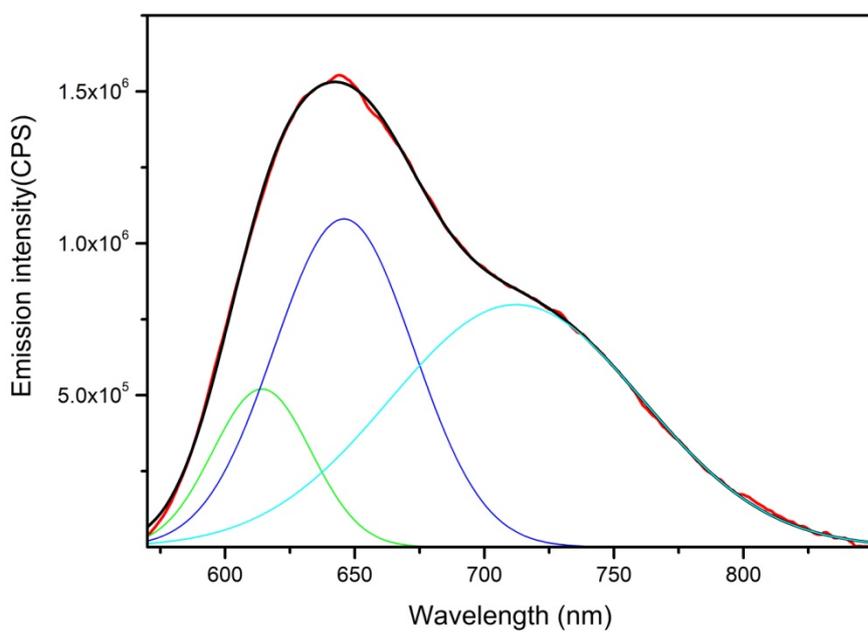


Figure S9: Deconvolution of the steady state emission spectra of a sample from *Codex Brixianus*: darker side (CB-DS- black line).

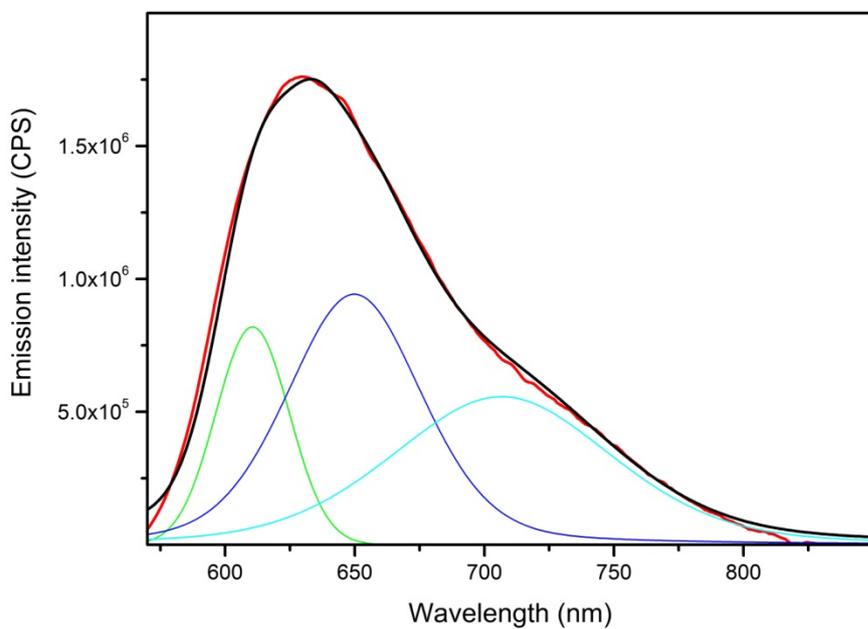


Figure S10: Deconvolution of the steady state emission spectra of a sample from *Codex Brixianus*: lighter side (CB-LS - red line).

Table S1: Wavelength values obtained as minima in the calculated second derivative and further used as input for deconvolution.

| | Wavelength (nm) | | | |
|------------|-----------------|-----|-----|-----|
| PF | 591 | 597 | 645 | 727 |
| PO | 597 | 617 | 645 | 726 |
| PM | 595 | 645 | 663 | 730 |
| POF | 597 | 645 | 725 | 790 |
| PFO | 599 | 645 | 730 | |
| CB | 605 | 645 | 727 | |

Highlights

- A non-invasive fluorimetric procedure for the detection of complex mixtures of purple dyes in historical codices has been developed.
- The characteristic spectroscopic features of orchil and folium on parchment has been determined.
- The purple dyes on a precious 6th century purple codex has been recognised.